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Short communication

Novel water soluble phosphonium chitosan derivatives: Synthesis, characterization and cytotoxicity studies

Ling Wang[a,1], Xiaofen Xu[a,1], Shengrong Guo[a,*], Zhaoxiang Peng[b], Tingting Tang[b,*]

[a] School of Pharmacy, Shanghai Jiao Tong University, 800th Dong-chuan Road, Shanghai 200240, China
[b] Shanghai Key Laboratory of Orthopaedic Implant, Department of Orthopaedic Surgery, Shanghai Ninth People's Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai 200011, China

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ABSTRACT

Novel water soluble phosphonium chitosan derivatives (WSPCSs) with two different degrees of substitution (3.6% and 4.2%) of quaternary phosphonium were synthesized in a homogeneous system at 25 °C. The chemical structures of the WSPCS were characterized by ^{31}P NMR (nuclear magnetic resonance), ^{1}H NMR, FT-IR (Fourier-transformed infrared) spectroscopy and WAXD (wide-angle X-ray diffraction). Their solubility in water and several organic solvents and cytotoxicity to L929 cells were also evaluated. The degree of substitution (DS) of WSPCS was calculated by ^{1}H NMR. WAXD analysis showed that the derivatives had low crystallinity. The derivatives could be easily dissolved in water. MTT (methyl thiazolyl tetrazolium) assay indicated that they had low cytotoxicity to L929 cells.

1. Introduction

Chitosan, poly [β-(1 → 4)-2-amino-2-deoxy-D-glucopyranose], is an amino polysaccharide obtained by the deacetylation of chitin. It is the second most abundant natural biopolymer and shows many excellent biological properties such as biodegradability, antimicrobial activity and immunological activity [1,2]. Recently, chitosan has been applied as a functional biomaterial for gene delivery [3–5], tissue engineering and so on [6,7]. However, the poor solubility of chitosan in common solvents except acidic aqueous solution restricted its application as a potential biomaterial. In order to improve the solubility of chitosan and broaden its application, chemical modification of chitosan [8–10], especially modifications with individual functional groups, is an effective strategy [11].

Quaternary chitosan derivatives, synthesized by introducing a functional quaternary ammonium moiety to chitosan [12–14], have improved the poor solubility of chitosan and have also shown great potential in various applications, such as in drug delivery systems [3] and gene delivery systems [15]. Recent studies show that cationic lipophilic quaternary phosphoniums have better antibacterial properties than quaternary ammoniums [16–18]. Chang et al. reported that the quaternized triphenylphosphonium-modified polyphenylene oxide (PPh_3-PPO) showed superior thermal stability as well as antibacterial activity against *Staphylococcus epidermidis* and *Escherichia coli*, whereas their ammonium analogs were only active against *S. epidermidis* [19]. It has also been found that some compounds with quaternary phosphonium groups have excellent anticancer activities [20–22]. In addition, quaternary phosphoniums, especially triphenylphosphonium ions, are effective mitochondria targeting groups. They have also been used for targeting anticancer drugs to tumor site-specific mitochondria [23] and targeting some antioxidants for the treatment of a range of degenerative diseases [24,25].

Chitosan derivatives with cationic lipophilic quaternary phosphoniums might play a better role than chitosan or conventional quaternary chitosan derivatives in the relevant applications. Until now, there is no report on quaternary phosphonium chitosan derivatives. Thus, the aim of this study is to prepare phosphonium chitosan derivatives (WSPCSs) with cationic lipophilic quaternary phosphoniums. The chemical structures of WSPCSs were characterized by FT-IR, ^{1}H NMR, ^{31}P NMR and WAXD. Their solubility and cytotoxicity to L929 cells were also investigated.

2. Experimental

2.1. Materials

Chitosan with a viscosity-average molecular weight of 200 kDa and 91.8% (DD %) N-deacetylation was purchased from Zhejiang Yuhuan Ocean Biochemistry Co., Ltd. (2-Carboxyethyl)

* Corresponding authors. Tel.: +86 21 34204793; fax: +86 21 34204793.
E-mail addresses: srguo@sjtu.edu.cn (S. Guo), tingtingtang@hotmail.com (T. Tang).
[1] These authors contributed equally to this study.

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triphenylphosphonium chloride (CTPC) was purchased from Alfa Aesar (Ward Hill, MA) with a purity of 98%. 1-Ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC·HCl) and 1-hydroxybenzotrizole (HOBt) were purchased from Shanghai Medpep Co., Ltd. Foetal bovine serum (FBS), penicillin and streptomycin were purchased from Beijing Solarbio Science & Technology Co., Ltd. α-MEM was obtained from GIBCO (Grand Island, NY, USA). Other chemicals used were of analytical grade. Ultra-pure water (>18.2 MΩ cm, Millipore Milli-Q system) was used in the experiments.

2.2. Preparation of WSPCS

Chitosan (0.2 g, 1.22 mmol of amino groups) was stirred with two molar equivalent of HOBt (0.332 g, 2.44 mmol) in 20 mL ultra-pure water at 25 °C until the homogenous solution was obtained. The solution was mixed with a solution of CTPC (0.452 g, 1.22 mmol) in ultra-pure water (5 mL) followed by dropwise adding a solution of EDC·HCl (0.47 g, 2.44 mmol) in ultra-pure water (5 mL). The reaction was carried out at 25 °C for 48 h. The yellowish reaction products were concentrated and poured into the mixed solvent which is made of methanol and diethyl ether (v/v, 1:2). Then the products were centrifuged and thoroughly washed with methanol and diethyl ether, following by drying under vacuum.

2.3. Characterization of WSPCS

2.3.1. FT-IR spectroscopy

Fourier-transformed infrared (FT-IR) spectra of Chitosan and WSPCS were recorded on Varian 640-IR Spectrophotometer. Samples were prepared as KBr pellet and were scanned against a blank KBr pellet background at wavelength range of 4000–600 cm^{-1} with resolution of 4.0 cm^{-1}.

2.3.2. ^{1}H NMR spectroscopy

^{1}H NMR spectrum of WSPCS in D_2O was obtained on a Varian Mercury Plus 400 spectrometer at 25 °C. Data collection consisted of 256 acquisitions.

2.3.3. ^{31}P NMR spectroscopy

^{31}P NMR spectra of CTPC and WSPCS in D_2O were obtained on the same spectrometer at 25 °C. Data collection consisted of 128 acquisitions for CTPC and 2000 acquisitions for WSPCS, respectively.

2.3.4. Wide-angle X-ray diffraction (WAXD)

Wide-angle X-ray diffraction (WAXD) was performed on an X-ray diffractometer (D/Max-2200/Pc, Rigaku, Japan) using fixed monochromatized Cu-Kα radiation (40 kV, 30 mA) with 5°/min scanning rate at room temperature. Diffraction was measured in a range of $2\theta = 5–55°$.

2.4. Estimation of solubility

The chitosan or WSPCS samples (100 mg) were added in 2 mL of ultra-pure water and kept shaking at 110 rpm in a water bath (25 °C) until dissolution equilibrium was reached. The soluble part of the sample was removed by centrifugation, and the undissolved part was dried in an oven under a vacuum and weighed. The solubility (S) was defined as:

$$S(\%) = \frac{100 - W_1}{2} \times 100$$

W_1: the weight of the undissolved part (mg)

The solubility of chitosan or WSPCS in other solvents was measured by the same method.

The pH dependence of water solubility of samples was evaluated using turbidity measurement. The test sample (0.2 g) was dissolved in 1% HAc (100 mL). With stepwise addition of NaOH solution (1 M), the transmittance of the solution was recorded with a UV (Spectrumlab 54) spectrophotometer at 600 nm.

2.5. Evaluation of cytotoxicity

All stock solutions were 1 mg/mL in pH5.5 HAc–NaAc buffer and diluted to different concentrations ranging from 5 to 200 μg/mL by the same buffer. L929 cells (mouse fibroblasts) were cultured in α-MEM medium supplemented with 10% FBS at 37 °C, 5% CO_2 and 95% relative humidity.

Cytotoxicity of chitosan and WSPCS was determined by MTT assay, which was adapted according to the method of Lu et al. [26]. L929 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. After 24 h, cell culture medium was aspirated and replaced by 100 μL serial dilutions of polymer solution in α-MEM medium without FBS. The cells were then incubated for 4 h at 37 °C. Afterwards, medium was replaced by 200 μL α-MEM without FBS containing 0.5 mg/mL MTT. After 4 h incubation at 37 °C in the dark, medium was removed and 200 μL DMSO was added. Plates were shaken evenly for 10 min. Measurement was performed using an ELISA plate reader (Varioskan Flash) at wavelength of 490 and 570 nm. Relative viability was calculated using 0% (wells without cells) and 100% (wells with polymer-untreated cells) as controls. Each value was averaged from 5 parallel experiments.

3. Results and discussion

3.1. Chitosan–HOBt system

As it is known, chitosan with high molecular weight could hardly dissolve in any solvents except acidic aqueous solutions, such as hydrochloric acid and acetic acid. The reason for the dissolution of chitosan is that the amino groups of chitosan are protonated by the protons in acid solution and the protonation destroys intermolecular and intramolecular hydrogen bonds of chitosan. Chitosan is completely dissolved by simply mixing chitosan with HOBt in water. One reason is that the pH of chitosan–HOBt solution is between 4 and 5. It was also observed that the water solubility of both HOBt and chitosan could be improved in the presence of each other. This suggests that there existed a certain interaction and complexation between chitosan and HOBt in the system. The possible mechanism of the interaction and complexation is shown in Fig. 1, which was also reported by Fangkangwanwong and Dai based on NMR study [27–29]. The complexes existing as salt form not only increased the water solubility of both chitosan and HOBt but also activated the amino groups of chitosan to react with the active ester of CTPC.

Fig. 1 presents the possible mechanism of the reaction of chitosan–HOBt complex and CTPC via EDC–HCl. Then CTPC was added into the chitosan–HOBt solution and EDC·HCl was dropped into the solution with continuous stirring. Once EDC was protonated by HCl, carbocations were formed. Some carbocations were hydrolyzed and others reacted with CTPC to generate O-acylisourea. If excessive EDC was added, N-acylisourea could be formed. Finally, O-acylisourea reacted with A or B and the coupling reaction probably occur at C-2 to form an amide linkage. It was noted that the HOBt used in chitosan–HOBt system could also reduce the amount of generated N-acylisourea and moderate the reaction rate of the coupling reaction [29].

3.2. FT-IR analysis

The FT-IR spectrum of chitosan and WSPCS (4.2%) is shown in Fig. 2(A). The peak at 1512 cm^{-1} was probably assigned to amide II